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FRACTIONATION OF TOXINS FROM 'HYDROPHIS CYANOCINCTUS' VENOM AND DETERMINATION OF AMINO ACID COMPOSITION AND END GROUPS OF HYDROPHITOXIN

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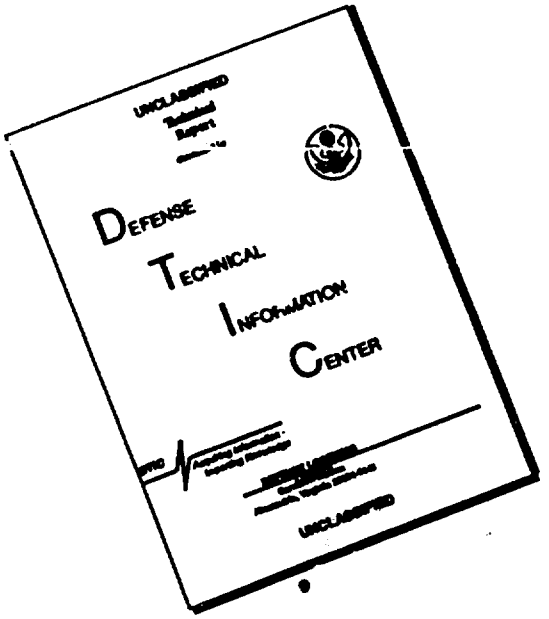

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13 ABSTRACT

THREE TOXIC FRACTIONS WERE SEPARATED FROM CRUDE VENOM OF THE SEA SNAKE HYDROPHIS CYANOCINCTUS BY COLUMN CHROMATOGRAPHY. THE MOST ABUNDANT FRACTION, CALLED HYDROPHITOXIN A, HAS BEEN STUDIED FOR AMINO ACID COMPOSITION AND FOUND TO CONTAIN 19 OF THE COMMON AMINO ACIDS (INCLUDING ASPARAGINE AND GLUTAMINE); PHENYLALANINE IS ABSENT. THE TOTAL NUMBER OF AMINO ACID RESIDUES PER MOLECULE IS ESTIMATED TO BE 59. METHIONINE OCCUPIES THE N-TERMINAL POSITION AND ASPARAGINE IS AT THE C-TERMINAL END. FROM THESE PRELIMINARY RESULTS HYDROPHITOXIN A APPEARS TO BE SIMILAR IN MOLECULAR SIZE AND STRUCTURE TO SEVERAL OTHER SEA SNAKE VENOMS PREVIOUSLY REPORTED.

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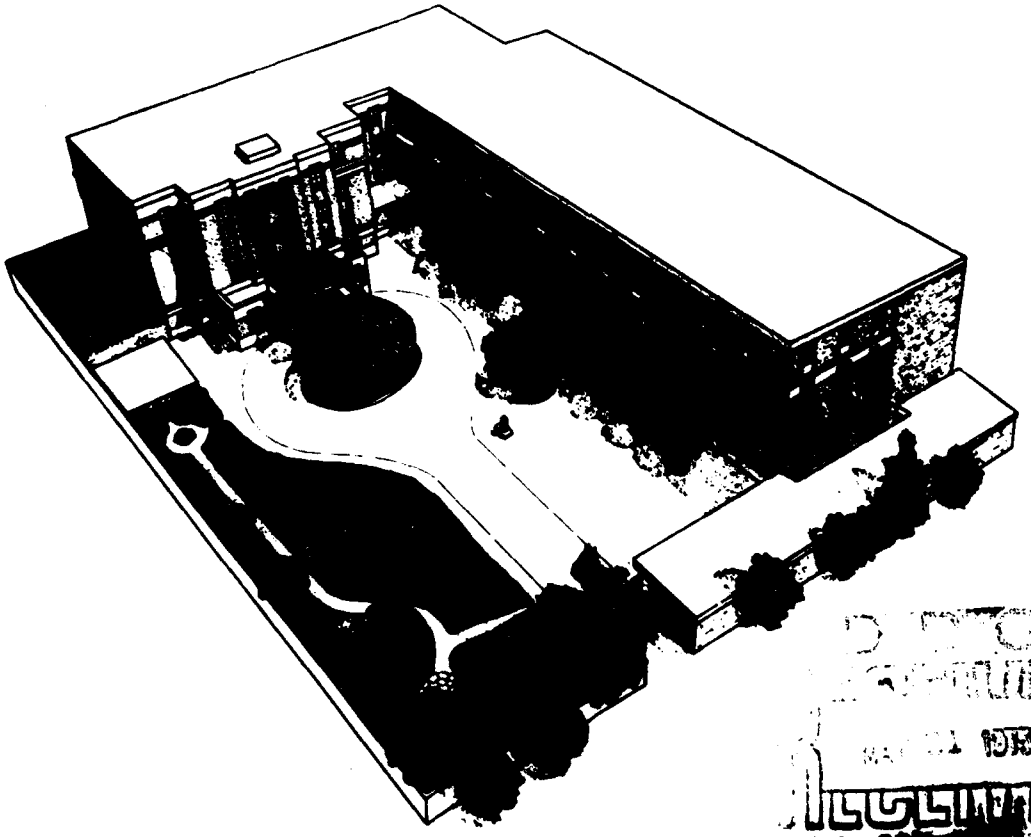

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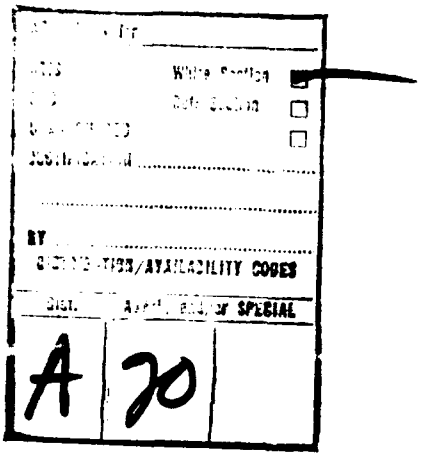

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FRACTIONATION OF TOXINS FROM *HYDROPHIS CYANOCINCTUS* VENOM AND DETERMINATION OF AMINO ACID COMPOSITION AND END GROUPS OF HYDROPHITOXIN a*

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Abstract—Three toxic fractions were separated from crude venom of the sea snake *Hydrophis cyanocinctus* by column chromatography. The most abundant fraction, called Hydrophitoxin a, has been studied for amino acid composition and found to contain 19 of the common amino acids (including asparagine and glutamine); phenylalanine is absent. The total number of amino acid residues per molecule is estimated to be 59. Methionine occupies the N-terminal position and asparagine is at the C-terminal end. From these preliminary results Hydrophitoxin a appears to be similar in molecular size and structure to several other sea snake venoms previously reported.

INTRODUCTION

VENOMS from several species of sea snakes have been studied recently (ENDO *et al.*, 1971; SATO and TAMIYA, 1971; SATO *et al.*, 1969; TAMIYA and ARAI, 1966; TU and HONG, 1971; TU *et al.*, 1971; TU and TOOM, 1971) despite the low amounts of venom produced by these snakes. The present preliminary report describes the separation of several toxic components from the crude venom of *Hydrophis cyanocinctus*, which is a common sea snake in Formosan waters. In addition, the amino acid composition and end groups have been determined for one purified protein fraction which has been named Hydrophitoxin a.

MATERIALS AND METHODS

Sea snakes of the species *Hydrophis cyanocinctus* were obtained from fishermen from various fishing ports in Taiwan and brought to our laboratory in Taipei. The snakes were maintained for several weeks in a sea water aquarium and venom collected into capillary tubes at approximately weekly intervals. It was difficult to maintain the snakes in a healthy state more than approximately one month under our laboratory conditions. The snakes, which averaged approximately 200 g in body weight, yielded approximately 1 mg of crude dried venom per weekly collection. The pooled fresh venom was lyophilized prior to further treatment.

The best separation of the crude venom into its component protein fractions was achieved by column chromatography on Whatman CM 52 carboxymethylcellulose. In a typical run,

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44 mg of lyophilized crude venom were dissolved in 0·2 ml of 0·01 M sodium phosphate buffer, pH 6·3, containing 0·01 M sodium chloride (TAMIYA and ARAI, 1966; SATO *et al.*, 1969) and applied to the carboxmethylcellulose-packed column (0·9×21 cm) which had been previously equilibrated with the same 0·01 M sodium phosphate–0·01 M sodium chloride buffer. Separation of the protein fractions was accomplished at pH 6·3 with an increasing sodium ion concentration gradient provided by mixing 500 ml of the initial phosphate buffer described above with 500 ml of a second 0·01 M phosphate buffer, pH 6·3, containing 0·50 M sodium chloride. The flow rate was maintained at 22 ml/hr and 1·8 ml were collected in each tube fraction. The tubes were checked spectrophotometrically at 280 nm.

Further purification of individual component proteins or polypeptides separated from the venom in the above procedure was achieved with initial desalting by passage through a Sephadex G-15 column with 0·1 M ammonium acetate solution at pH 6·7. The Sephadex column had been pre-treated by the method of GOODSON and DISTEFANO (1969) to improve the resolution. This treatment was followed by subjecting the desalted material to a second fractionation by gradient elution through a column packed, as before, with CM-52 carboxymethylcellulose but using a different gradient system provided by increasing concentrations of ammonium acetate. The gradient elution system was comprised of eight 125 ml portions of ammonium acetate solutions, at pH 6·7 and ranging in 0·01 M increments from 0·10 M to 0·17 M, which were placed in the eight compartments of a Technicon Autograd gradient device.

Samples were examined at intervals with cellogel electrophoresis using 0·05 M ammonium acetate buffer, pH 6·7; staining of the polypeptide and protein bands was done with 0·5 per cent Ponceau-S dye in 20 per cent trichloroacetic acid (the high concentration of trichloroacetic acid was used for fixative). Disc electrophoresis also was used in the usual way.

Carboxymethylation of half-cystine groups in one fraction of the venom was carried out by the method of CRESTFIELD, MOORE and STEIN (1963). The purified material first was reduced at room temperature for 4 hr with β-mercaptoethanol in an 8 M urea solution containing tris buffer at pH 8·6; this treatment was followed by reaction for 30 min at room temperature with idoacetic acid in 1 M sodium hydroxide. Desalting of the carboxymethylated compound was carried out by passage through a Biogel P-2 column (50–100 mesh) using 0·2 M acetic acid. The column was wrapped in aluminium foil to prevent interference from light.

Purified polypeptide fractions were hydrolyzed for amino acid composition analysis by use of 6 M hydrochloric acid in sealed evacuated tubes for 12, 24 and 72 hr intervals at 110°C. In the first few hydrolyses, phenol in 9 mg per cent concentration was added to the solutions to protect the tyrosyl groups; later, mercaptoethanol 1:2000 v/v was used to protect serine, threonine, tyrosine and methionine by the method of KEUTMANN and POTTS (1969). Amino acid analyses of hydrolysates were made with a Technicon Amino Acid Analyzer. Tryptophan was determined in purified fractions spectrophotometrically by the method of GOODWIN and MORTON (1946) and BEAVEN and HOLIDAY (1952).

One purified fraction was treated with trypsin in 0·1 M ammonium bicarbonate solution for 3 hr at 37°C The resulting mixture of peptides were studied by peptide mapping using our modification (BLACKWELL *et al.*, 1969) of procedures described by INGRAM (1958) and BAGLIONI (1961).

Selected determinations of C-terminal amino acids were made using Carboxypeptidase A (Worthington premium quality enzyme WCOA 9KA), carried out in 0·2 M ammonium bicarbonate solution for 24 hr at 37°C. N-terminal amino acids were analyzed using dansylation procedures as outlined by HARTLEY (1970) and the PTH method of EDMAN (1950) as described

by IWANAGA *et al.* (1969). PTH-amino acids were identified with thin-layer chromatography using pre-coated TLC plate silica gel F-254 (E. Merck) according to the method of JEPPSSON and SJÖQUIST (1967). Dansyl-amino acid derivatives were released after 4 hr hydrolysis aa recommended by GROS and LABOUESSE (1969) and were identified on polyamide thin layer as described by HARTLEY (1970).

Preliminary toxicity studies were made on various fractions produced during the separation and purification of the crude venom. In most cases each test used five 20 g mice which were injected intraperitoneally; 50 microgram quantities of test material were used and the time required for death of the animal measured (G. S. Huber, unpublished data).

RESULTS AND DISCUSSION

The principal protein fractions obtained by the initial fractionation of the crude venom on the carboxymethylcellulose column are illustrated in Fig. 1. Eight peaks were present in which the optical density exceeded 0·1. Preliminary toxicity studies were made on each of the eight fractions; although insufficient studies were made to establish reliable LD_{50} dosages, Fractions VI, VII and VIII possessed relatively high levels of neurotoxicity.

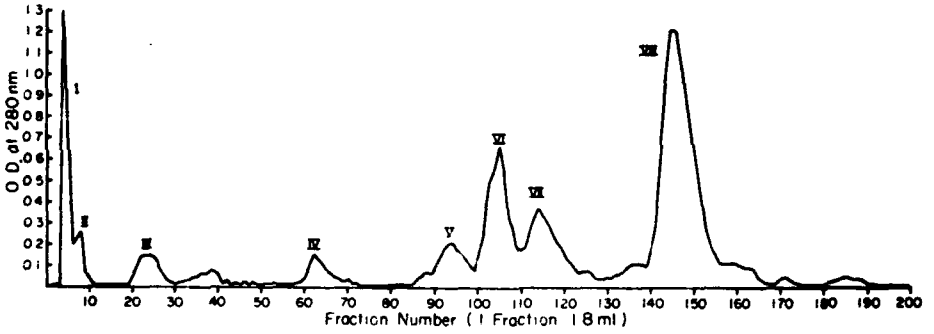

FIG. 1. FRACTIONATION OF *Hydrophis cyanocinctus* VENOM BY CARBOXYMETHYLCELLULOSE COLUMN CHROMATOGRAPHY WITH SODIUM CHLORIDE GRADIENT.

The toxic material which was present in largest amount was contained in Fraction VIII. This fraction, after lyophilization, desalting by passage through a Sephadex G-15 column at pH 6·7 and drying, was re-fractionated by gradient elution on carboxymethylcellulose as described above using ammonium acetate solutions at pH 6·7. One major and one adjoining minor peak were obtained as shown in Fig. 2.

The material in the central portion of the major peak (from tube fractions 124 to 140) was taken for further study. On cellogel electrophoresis the material appeared to be relatively homogeneous and pure, as shown in Fig. 3. Disc electrophoresis showed a minor band of impurity which was considered to be too small to interfere with structural analysis. Therefore this purified material, named Hydrophitoxin a, was used for structure studies.

Ten mg of the Hydrophitoxin a were carboxymethylated (CRESTFIELD *et al.*, 1963) and desalted by passage through a Biogel P-2 column. Several 0·1 mg samples of the dried product were hydrolyzed and analyzed quantitatively for amino acids. Results from 4 separate 24-hr hydrolysates are summarized in Table 1 which also gives the deduced number of residues of each amino acid found. All of the common amino acids were present except phenylalanine.

TABLE 1. AMINO ACID COMPOSITION OF HYDROPHITOXIN a

	Sample 1*		Sample 2*		Sample 3†		Sample 4†		
	nMoles	Molar ratio	nMoles	Molar ratio	nMoles	Molar ratio	nMoles	Molar ratio	Nearest integer
CM-Cys	150	7·5	45	6·4	72	8·3	60	8·4	8
Asp	120	5·5	36	5·1	48	5·6	43	6·0	6
Thr	140	6·4	41	5·9	56	6·5	47	6·6	7
Ser	100	4·5	31	4·4	39	4·5	34	4·7	5
Glu	175	8·0	54	7·7	67	7·7	58	8·1	8
Pro	34	1·5	9·0	1·3	15	1·7	11	1·5	2
Gly	90	4·1	29	4·1	35	4·0	31	4·3	4
Ala	22	1·0	7·5	1·1	8·2	0·95	7·2	1·0	1
Val	20	0·91	5·5	0·79	7·1	0·83	5·7	0·79	1
Met	16	0·73	5·0	0·71	6·4	0·74	5·6	0·78	1
Ile	41	1·9	12	1·7	14	1·6	11	1·5	2
Leu	23	1·0	6·5	0·93	9·8	1·1	9·6	1·3	1
Tyr	20	0·91	5·0	0·71	8·9	1·0	6·4	0·90	1
Lys	140	6·4	43	6·1	52	6·0	43	6·0	6
His	45	2·0	14	2·0	18	2·1	14	2·0	2
Arg	63	2·9	19	2·7	28	3·2	23	3·2	3
Trp‡									1

*Sample 1 and sample 2 were hydrolyzed for 24 hr at 110°C with constant boiling HCl including 9 mg per cent phenol concentration.

†Sample 3 and sample 4 were hydrolyzed for 24 hr at 110°C with constant boiling HCl with mercaptoethanol in 1:2000 v/v as mentioned by KEUTMANN and POTTS (1969). Higher recoveries of threonine, serine, methionine and tyrosine were observed.

‡Tryptophan was estimated spectrophotometrically by the method of Goodwin and Morton and calculated by the formula derived by Beaven and Holiday. The ratio of Trp/Tyr equalled 1·1.

TABLE 2. COMPARISON OF NUMBERS OF AMINO ACIDS IN HYDROPHITOXIN a WITH THOSE IN ITS TRYPTIC PEPTIDES

Amino acid residue	From analysis of toxin	Tryptic peptides										
		T-1	(T-2)*	T-3	T-4	T-5	T-6	T-7	T-8	(T-9)*	T-10	(T-11)†
CM-Cys	8	3	2	2	1	2						
Asp	6	3	1		1	1		1				
Thr	7	1	3		1	3		1	1			
Ser	5		2		2	2		1				
Glu	8	2	1	1	3	1	1					
Pro	2			1	1							
Gly	4			2					1	1	1	
Ala	1		1			1						
Val	1			1								
Met	1				1							
Ile	2						1			1	1	
Leu	1	1										
Tyr	1		1			1						
Lys	6		1	1	1	2				1	2	1
His	2	1						1				
Arg	3						1	1	1			
Trp	1							1‡				
Total	59	11	(12)	8	11	13	3	6	3	(3)	4	(1)

*(T-2)+Lys=T-5 and (T-9)+Lys=T-10.
†T-11 contained only lysine.
‡Positive to Ehrlich stain.

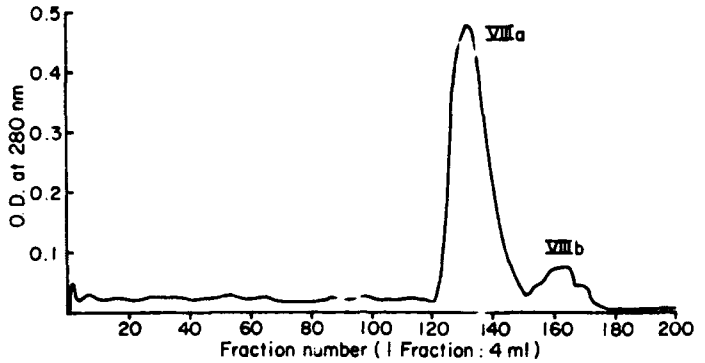

FIG. 2. FURTHER PURIFICATION OF THE PRINCIPAL TOXIC MATERIAL (FRACTION VIII FROM FIG. 1) BY CARBOXYMETHYLCELLULOSE COLUMN CHROMATOGRAPHY WITH AMMONIUM ACETATE GRADIENT.

The presence of one tryptophan residue per molecule of toxin was estimated spectrophotometrically. From these combined results our estimate of the total number of amino acid residues per molecule is 59.

The pattern of peptides released by tryptic digestion also was studied by peptide mapping. The pattern, illustrated in Fig. 4, showed eleven principal peptide spots after ninhydrin-staining. Material was eluted separately from each of the eleven spots from several similar peptide maps, hydrolyzed, and analyzed quantitatively for amino acid content. The results of the analyses are summarized in Table 2. All 59 of the amino acid residues are accounted for in eight of the eleven peptides; two other peptides, T-2 and T-9, represent respective duplications of peptides T-5 and T-10 which each contain two lysine residues. "Peptide" T-11 contained only lysine. The electrophoretic mobilities of the eleven peptides were compatible with their amino acid compositions.

By Carboxypeptidase A digestion of Hydrophitoxin a in 0·2 M ammonium bicarbonate solution at 37°C for 24 hr the C-terminal amino acid residue was found to be asparagine. Dansylation (HARTLEY, 1970) and PTH amino acid derivatives were used to identify the N-terminal amino acid which was found to be methionine. It is of interest that methionine also was found by BOTES (1971) in the N-terminal position of toxin β from *Naja nivea* venom.

Detailed structural comparison of Hydrophitoxin a with the neurotoxins from other sea snakes and land snakes must await further work including amino acid sequence studies now in progress; however, it appears to be similar in composition to those reported in several other species (TU and HONG, 1971; YANG *et al.*, 1969) and most similar to the toxin reported in the venom of *Lapemis hardwickii* (TU and HONG, 1971).

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FIG. 3. ELECTROPHORESIS OF PURIFIED TOXIN, HYDROPHITOXIN a (FROM FRACTION VIIIa IN FIG. 2) AND CRUDE VENOM (BOTTOM POSITION IN FIGURE) ON CELLOGEL.

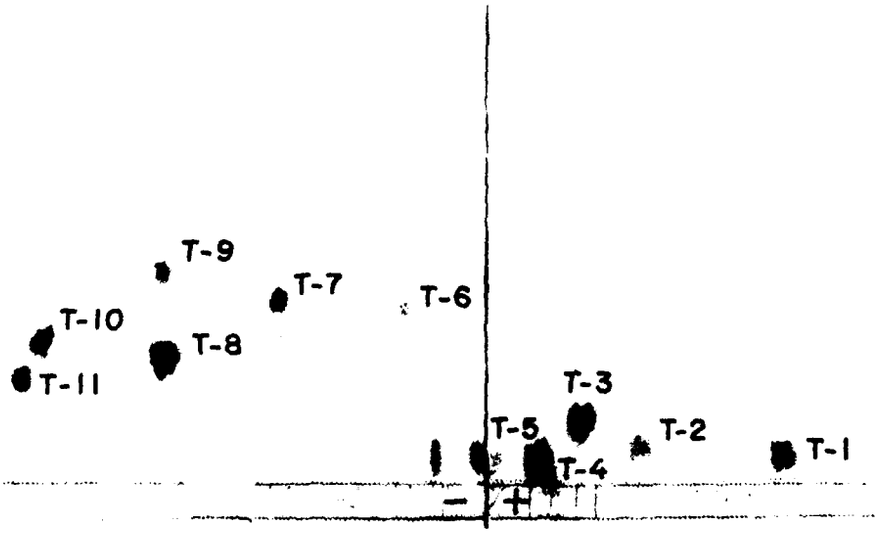

FIG. 4. MAP OF PEPTIDE MIXTURE PRODUCED BY TRYPTIC DIGESTION OF HYDROPHITOXIN a.

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